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In vitro 3D bone tissue models, from cells to controlled and dynamic environment

Guenaelle Bouet¹, David Marchat^{2}, Magali Cruel³, Luc Malaval¹, Laurence Vico¹*

¹ *Laboratoire de Biologie du Tissu Osseux and Institut National de la Santé et de la Recherche Médicale - U1059, Université de Lyon - Université Jean Monnet, Saint-Etienne, France*

² *Center for Biomedical and Healthcare Engineering, Ecole Nationale Supérieure des Mines, CIS-EMSE, CNRS:UMR 5307, F-42023 158 cours Fauriel Saint-Etienne cedex 2, France*

³ *University of Lyon, LTDS, UMR CNRS 5513, Ecole Centrale de Lyon, 36 avenue Guy de Collongue, 69134 Ecully Cedex, France*

Full mailing address and contact information for EACH author:

** Corresponding author: David Marchat, Tel.: +33 4 77 49 97 01; fax: +33 4 77 49 96 94. E-mail: marchat@emse.fr*

Guenaelle Bouet, Tel.: +33 4 77 42 14 47; fax: +33 4 77 57 55 72. E-mail: guenaelle.bouet@gmail.com

Magali Cruel, Tel.: +33 4 72 18 62 15; fax: +33 4 77 49 96 94. E-mail: magali.cruel@ec-lyon.fr

Luc Malaval, Tel.: +33 4 77 42 14 44; fax: +33 4 77 57 55 72. E-mail: luc.malaval@univ-st-etienne.fr

Laurence Vico, Tel.: +33 4 77 42 18 57; fax: +33 4 77 57 55 72. E-mail: vico@univ-st-etienne.fr

Abstract:

Most of our knowledge of bone cell physiology is derived from experiments carried out *in vitro* on polystyrene substrates. However, these traditional monolayer cell cultures do not reproduce the complex and dynamic 3-dimensional (3D) environment experienced by cells *in vivo*. Thus, there is a growing interest in the use of 3D culture systems as tools for understanding bone biology. These *in vitro* engineered systems, less complex than *in vivo* models, should ultimately recapitulate and control the main biophysical, biochemical and biomechanical cues that define the *in vivo* bone environment, while allowing their monitoring. This review focuses on state of the art and the current advances in the development of 3D culture systems for bone biology research. It describes more specifically advantages related to the use of such systems, and details main characteristics and challenges associated with its three main components, i.e. scaffold, cells and perfusion bioreactor systems. Finally, future challenges for non-invasive imaging technologies are addressed.

Keywords: Bone tissue engineering, Perfusion bioreactor, Cell culture, Scaffold, Experimental approaches, Mechanical stimuli

1. CONTEXT

1.1. Tissue Engineering: concepts

Tissue engineering is the application of the principles of biology and engineering to the development of artificial living tissues (1). It utilises specific combinations of cells, matrices (referred also as “scaffolds or constructs”), as well as cellular, chemical and mechanical signals. Generally speaking, current applications of tissue engineering can be divided into two categories.

The first category consists of elaborating biological substitutes to restore, maintain or improve tissue functions (1). This therapeutic approach aims to replace/repair damaged human tissues with manufactured tissue-engineered products. This field is quickly expanding due to coordinated and converging technological developments in the creation and/or manipulation of biomolecules, biological materials, cells and tissues, with the goal of ultimately generating pseudo-tissues and organs for transplant. Such therapy will be drastically different from classical prosthesis implants, avoiding integration, interface and wearing-out problems, thus allowing for a longer lived tissue/organ substitution.

The second category of applications of tissue engineering aims to understand the fundamental aspects of cells working *in vivo* in 3D controlled systems. In fact, *in vivo* cells reside in a complex three dimensional (3D) micro-environment, encompassing several cell types producing/exchanging many signals, interacting in a dynamic fashion amongst themselves. These cells also interact with an extracellular matrix whose rigidity varies in time and space with the nature and/or maturity of the cell type/tissue (2, 3). Moreover, in tissues like bone, cells experience various mechanical stresses (e.g. compression forces). Therefore, *in vitro* 3D systems, based on tissue engineering principles, strive to reproduce (at least partly) the *in vivo* environment of cells in a scaffold material. As it was recently

demonstrated by many studies (4, 5) for review), such systems implies the use of perfusion bioreactors to control and monitor biochemical and mechanical signals throughout cell culture.

Such complex models represent the best approach to understand the bone (cell) biology since they allow study of a multicellular microenvironment whose structure, composition, topology and perfusion conditions are adaptable and more realistic, respective to the *in vivo* situation, than the current two dimensional (2D) standard cell culture method. For instance, in these systems it will be possible to test the effects of chemical substances or characterise stimuli acting locally on cell metabolism in order to understand cellular and matrix changes involved in aging mechanisms and some diseases (e.g. osteoporosis); they will also provide tools for assessing drugs in development in toxicology or pharmacology studies.

The knowledge gained from such experiments will advance our understanding of pathophysiology as well as the prevention, diagnosis and treatment of diseases.

1.2. From 2D in vitro cell culture to native bone tissue, the missing link

Bone tissue development and remodeling in living organisms are orchestrated by cascades of regulatory factors interacting at multiple levels, in both time and space.

Animal models provide the full complexity of biological systems (at least within a given species), but they offer limited control of the local environment and scanty real time information. In contrast, traditional cell culture in 2D allows significant control of the cellular environment and a direct access to cellular processes. However, these culture conditions are drastically simplified and hardly reproduce the typical bone environment in the organ/organism, in which (bone) cells develop in a 3D structure subjected to mechanical stimulation. Moreover, it is well established that signal transduction and many other cellular

functions differ between 2D and 3D culture conditions (6-8). These differences are expected since on 2D substrates the cells have to adapt to an artificial flat and rigid surface. For instance, Elsdale et al have shown that morphological changes in fibroblasts cultivated onto 2D plastic or in 3D collagen gels are different. More precisely, cells cultivated in 3D gels are more similar to connective tissue cells *in vivo* than on 2D (9). These results highlight the relevance of 3D systems to reproduce *in vivo* environment.

Cultivating cells in a scaffold provides 3D architecture and potentially mechanical stimulation, but it is only the first step towards 3D *in vitro* models reproducing the *in vivo* environment for long-term studies. Indeed, cells seeded in scaffolds and cultivated under static condition (in multiple well plates) tend to reach high concentration at the scaffold periphery, resulting in poor nutrient and waste exchange in the middle of the scaffold and thus cell necrosis in the center of the scaffold. Perfusion bioreactors were designed to avoid this drawback and establish a closely monitored and tightly controlled environment. The perfusion also permits to mechanically stimulate the cells via shear stress (10) for review). These bioreactors can be improved with the addition of a compression system dissociated from or combined with perfusion (11), which is particularly useful and relevant in bone cell studies (e.g. analysis of mechanotransduction (12)).

Three-dimensional artificial models can thus overcome the limitation of current 2D models by providing robust spatial and temporal control of biophysical, biochemical and mechanical micro-environmental cues (13-15). Additionally, the development of such alternative methods follows the ethical regulation drive to reduce the use of animal models.

In this review, we provide an overview of the main concepts, key points and challenges about *in vitro* 3D cultures dedicated to experimental/fundamental research in bone biology.

In particular, it focuses on the four main components of such strategy: the scaffold, the cells, the bioreactor and the none-invasive imaging technologies.

2. SCAFFOLD

2.1. Why use scaffolds?

As mentioned previously, *in vitro* bone development requires 3D organization to simulate the structure, mechanical properties (i.e. stiffness and strength), composition as well as regulatory cues of the natural ECM (16, 17). 3D artificial models may provide a robust spatial and temporal monitoring of biophysical, biochemical and mechanical micro-environmental cues; this monitoring is unavailable *in vivo* (14). As such, they are crucial tools for identifying regulators and pathways of bone cell behavior and fate.

2.2. Ideal scaffold properties

While the success of an implanted scaffold *in vivo* will be based on its capacity to be invaded, degraded then replaced by host tissue, the purpose of a scaffold for *in vitro* experimental application is to provide a structural support for the culture of cells in a controlled 3D environment *in vitro*. This environment should meet as many as possible of the requirements of the cells used, and ideally mimic the dynamic native niche environment of, e.g. stem cells (18, 19). Even though there are no unique scaffold specifications for such experimental applications, scaffold needs to be at least biocompatible and osteoconductive to support cell attachment, growth and differentiation (20). Moreover, although the final features depend on the specific purpose of the *in vitro* study, several general characteristics and requirements need to be considered for all designs. Besides stiffness (14, 21), scaffolds must have adequate mechanical properties to support, if applicable, flow perfusion and

mechanical stimuli (e.g. compressive strength). Scaffolds for *in vitro* use do not necessarily have to be degradable (i.e. soluble and/or resorbable). However, the phase and chemical composition, as well as surface chemistry (cell-surface interface), are key parameters that affect cellular responses (22, 23). Similarly, architecture influences the interactions between the cells, the media and the material, ultimately controlling the rate and quality of new tissue formation (e.g. extracellular matrix deposition) (14). Both architecture and composition are very important features for this application (24) and are detailed in following subsections.

2.3. Architecture: A key role

For a 3D scaffold-based *in vitro* culture strategy, the scaffold architecture has to allow controlled and homogeneous cell seeding (25), cell response (26), mass transport (27), substrate degradation (28), and mechanical properties (29) throughout the experiment. The scaffold architecture (e.g. geometry, shape, size; as defined below) influences the cell fate both directly (e.g. nutrient accessibility) and indirectly (e.g. adsorption of proteins, local shear stresses). Macroscopic and microscopic architectural features are distinct (**Fig. 1**). They are generally defined as pore sizes greater than 100 μm and below 20 μm , respectively (for review (30)). Numerous studies have investigated the influence of macro- and micro-architecture on cell response and fate, and both were proved to be of great importance (14).

Macro-scale architecture can be split into macroscopic design (geometric structure) and internal pore structure/architecture, including the shape, size, distribution and interconnection of the macropores. Similarly, the micro-scale architecture consists in the shape (i.e. morphology), size (width and length), orientation (according to the way of perfusion flow for instance) and distribution (ordered vs random) of the micropores.

Since the 1990s, studies have emphasized the crucial effects of the micro-scale architecture of the constructs on cell seeding and response (26, 31, for review (32)). Micro-architecture has been shown to improve scaffold osteoconductivity (33, 34) and cause intrinsic osteoinductive activity (35, 36). These modification may be explained by (i) selective adsorption of proteins from biological fluids (37, 38), (ii) increase in specific surface area, promoting dynamic exchange at the biomaterial-matrix-cell interface (39), or (iii) the responsiveness of cells to substrate discontinuities (40, 41). For a comprehensive review focused on calcium phosphate ceramics, see (23).

According to the literature, the macro-scale architecture influences internal mass-transports (e.g. supply of nutrients, removal of deleterious waste compounds) (42), cell invasion/infiltration (e.g. seeding) (25, 43), tissue ingrowth (e.g. bone, blood-vessel) (44, 45), scaffold degradation (46, 47), inflammatory response (48), shear stress distribution (49, 50), and extensive mechanical properties of the scaffold (e.g. stiffness, compressive strength) (51). Likewise, the mechanical properties of the scaffold affect the intrinsic mechanical stimuli developed at the macroscopic (51) and microscopic cell level (49, 52) which, in turn, modify cellular response (20, 27, 53).

Macropore size has been shown to influence bone cell response *in vitro* and *in vivo* (54, 55). However, from the literature its effects appear similar for sizes ranging from 100 to 1200 μm (56, 57). The reason for this lack of logic are most likely the pore size (and shape) uncertainty due to the random internal pore architecture/morphology of the scaffolds used, and the method used to assess macropore and interconnection size (and shape) (58). This indicates that most studies do not exert sufficient control of the macroporous architecture to ensure cell response homogeneity (i.e. cell response related to perfectly defined architectural

features), As a consequence, the “mean macropore size” (i.e. macropores and interconnections) remains a confounding variable (59), even though it appears as the main macroscopic determinant of the biophysical properties of scaffolds (25, 26).

Advancements in engineering and materials has allowed for subtle tuning of scaffold features (e.g. macro-, micro-topography and surface chemistry). These improvements provided the opportunity to more accurately control the biophysical and biochemical micro-environmental cues in 3D biomaterial-based culture systems (13, 60). Many engineering methods are used to design and produce 3D scaffolds for tissue engineering applications, including porogen leaching (61), phase separation (61), gas foaming (26), replication of polymer template (62, 63), bone machining (64), and others (65, 66). Even though the internal scaffold architectures produced from these conventional methods can be modified by changing the fabrication parameters (synthetic materials), or can provide anatomical features with macro- and micro-environmental cues (natural based materials) (24), they suffer from sample-to-sample variations (e.g. random pore distribution and size) and design limitations. Unfortunately, the limits of these conventional methods prevent the structure standardization, as well as the experiment reproducibility; so many detrimental points for the creation of *in vitro* physiological models. Indeed, biological observations (67), physical (68) and mechanical (69) features, or simulation based on reconstructed images (70, 71) are, in that case, valid for one scaffold but not one architecture, diminishing the predictability of results. However, new engineering developments combining computational methods, such as computer-aided design (CAD), and additive manufacturing technologies (AM) (72) are able to overcome these production limitation by providing higher level of control over the design of manufactured scaffolds, with customized external shapes as well as optimized and reproducible internal morphologies (e.g. pore interconnectivity, shape, size and distribution)

(25, 73, 74). Recent reviews provide exhaustive classification of the different AM techniques, design methodologies and their limitations for production of scaffold made of different materials (60, 75, 76). Additionally, Giannitelli et al. reviewed studies relating the influence of some intrinsic architectural features of scaffold (e.g. porous design, pore morphology), controlled by AM fabrication parameters, on their extrinsic properties (e.g. shear stress level and distribution, mass transport, biological behavior) related for instance to the selected cell type grown or the fluid flow perfusion characteristics (75). A conclusion is that a design strategy is needed to match the micro and macro-scale intrinsic architectural features of the scaffold to the conflicting mechanical, mass transfer and biological requirements that it has to fulfil in a given experimental setup. One way to achieve scaffolds with specific target properties is to use computational tools/models to design their structure and AM process for their production (75). This strategy has been used to optimize scaffold design for mass transport (77), mechanical load (78, 79), or both (80, 81), shear stress at the surface of the pore walls (49, 50) and scaffold degradation (21).

Nevertheless, standard empirical approaches are not obsolete, quite the contrary! Recently, Bidan et al. proposed an elegant simple geometric model explaining the shape-dependence of growth in terms of tension and local curvature from static *in vitro* cell culture experiments and AM HA ceramics (82). In addition to confirm the model in a second article, they cleverly demonstrated that tissue growth into a porous scaffold is a function of pore geometry, specifically the local surface curvature (83); for other trial-and-error assessments on the influence of macroporosity, see (84, 85).

Also, beyond the control of the initial scaffold properties, especially permeability and mechanical strength, it is also important to anticipate their evolution during *in vitro*

experiments since scaffold degradation processes (e.g. dissolution, resorption, cracks) as well as neo-tissue ingrowth can significantly affect them (28).

Finally, the current limitations of the manufacturing processes result in geometrical discrepancies, generally anisotropic, between the fabricated and the designed scaffold (77, 81). In order to evaluate qualitatively how the fabricated scaffold matches the designed structure, scaffolds should be analyzed by high resolution imaging techniques such as micro-computed tomography (μ CT, see section 5). Quantitatively, the high resolution μ CT data should be used as input in the simulation in order to determine how these differences of geometry will influence the final scaffold properties (e.g. permeability) (86).

2.4. Scaffold composition

Material composition encompasses phase composition (i.e. amorphous or crystalline) and chemical composition (i.e. arrangement, type and ratio of atoms in material). Both associated to the chemical, mechanical and/or thermal treatments experienced by material during the manufacturing process govern the intrinsic chemical (e.g. thermodynamical solubility, hydrolysis susceptibility, chemical surface functional groups), physical (e.g. crystalline structure, wettability, surface charge), mechanical (e.g. stiffness, mechanical strength) and biological (e.g. toxicity, cell attachment) properties of scaffold. These intrinsic properties affect in turn the key early event of protein adsorption and subsequent cell adhesion, growth, differentiation, matrix deposition and function. Indeed, besides microarchitectural features such as roughness (see previous section), the type, conformation and density of adsorbed proteins depend mainly on the surface chemical functional groups, and on the ionic environment and pH value of the culture medium (for review (23, 86)). The latter can be significantly affected by the degradation mechanisms of the material. *In vitro*,

scaffolds can degrade via solution-mediated mechanisms (e.g. dissolution, hydrolysis), cell-mediated mechanisms (e.g. resorption, phagocytosis), as well as the loss of mechanical integrity (e.g. surface cracking) (87, 88). As degradation products can affect cell fate, they must also exhibit biocompatibility.

From a thermodynamic standpoint most calcium phosphates ceramics are faintly soluble in aqueous media, since their solubility products are low (89). However, John et al. emphasized a clear difference in MG-63 osteoblast-like cells attachment, spreading and growth between the quite insoluble hydroxyapatite (HA) and the more soluble β tricalcium phosphate (β TCP) ceramics, the former promoting cell adhesion and spreading and not the latter (90). These authors attributed the death of cell on β TCP ceramics to the high phosphate and low Ca levels in the material/media interface, resulting from dissolution/reprecipitation mechanism. An equivalent conclusion was reached by Detsch et al. to explain the inhibition of osteoclastic resorption (human monocyte-like cell line U-937) on highly soluble β TCP surface (91). Strangely, HA exhibits, as β TCP, negligible degree of resorption as well as low rate of bone ingrowth, despite its limited solubility compare to β TCP (91, 92). Thus, even if the degradation products of calcium phosphate bioceramics (calcium and phosphate ions) are naturally metabolized (93), their concentrations affect cells fate directly (e.g. cell death) and indirectly (e.g. proteins adsorption). These observations highlight that cell responses are very sensitive to the environment, and indicate that the performance of HA and TCP phases are not optimal. A relevant way to modulate/adjust the intrinsic properties of calcium phosphate (CaP) bioceramics, including solubility, and in turn the biological response consists of a partial replacement of the original constituting ions of the CaP lattice. For instance, the beneficial effects of carbonate ion substitution for phosphate and/or hydroxide

ions in HA lattice are widely established (92, for review (94)). Thus, managing carbonate content permits control of the rate of resorption and dissolution of synthetic carbonated hydroxyapatite (CHA) ceramics, which in turn enhance their bioactivity when compared to HA.

Biodegradation of synthetic polymers releases a mixture of oligomers, monomers and other low-molecular products. The rate of degradation is governed by intrinsic properties of the polymer such as molecular weight, chemical structure, copolymer ratio, crystallinity and the proportion of unstable bonds (for review (95)). The degradation products can include harmful or acidic byproducts (e.g. PLA, PGA, puramatrix hydrogel) which are harmful to cell function/survival (95, 96). In parallel, other physical and chemical surface properties of biodegradable polymers appear to be detrimental for tissue formation such as hydrophobic surfaces (97) or low cell adhesion capacity (98).

Cell fate is also driven by the mechanical properties of the scaffold and more particularly its stiffness. As in native tissue, cells within a construct respond and adapt to the mechanical and biological stimuli to which they are exposed (18, 99). It has been demonstrated by Engler et al., on polyacrylamide gels, that the substrate-level elasticity can specify the lineage specification of Mesenchymal Stem Cells (MSC, see section 3.1) (100). These results were confirmed recently by Zouani et al. (101); see also for review about effect of substrate rigidity on stem cells (102). Thus, scaffold used as hard tissue device, like bone, should exhibit high elastic modulus (103). Consequently, CaP ceramics, which have a stiffness similar to bone (23), have long been used for bone tissue engineering applications. In addition to considerable stiffness, CaP ceramics show biocompatibility, tunable bioactivity and biodegradability (94), and generally more bioactivity than synthetic polymers (104, 105). Yet, the brittle nature of these ceramics is a major issue especially for the application of

mechanical loading in a bioreactor (see section 4). However, this mechanical deficiency could be partly compensated by phase composition adjustment (85) and “topological optimization” (see section 2.3).

The alternatives to inorganic materials for the composition of scaffolds are mainly synthetic organic polymers, as naturally derived polymers suffer high batch-to-batch variability (95). The main advantage of polymers over ceramic, is that scaffolds can be easily produce with tunable micro- and macroscale features. Unfortunately, most porous polymeric scaffolds lack sufficient mechanical strength to withstand mechanical loading (for review (106)).

Combination of inorganic and organic components in a composite biomaterial could overcome their respective limitations, but it would make it extremely difficult to relate the scaffold physico-chemical properties to biological responses within the 3D model. Other materials such as metals (for review (107)) or carbon nanotubes (for review (108)) can display interesting properties for the development of 3D artificial models, provided that their toxicity and interaction with the biological *in vitro* environment (e.g. cells, proteins) can be controlled.

The development of 3D *in vitro* models requires to know the influence of scaffold chemistry (e.g. composition, surface chemistry) on cell behavior, and reciprocally to anticipate the influence of culture medium chemistry and cell activity on the scaffold. Thus, an ill-defined scaffold (i.e. not accurately characterized) will never permit understanding and control in reproducible manner the interaction between cells and substrate through adsorbed proteins (98). Therefore, batch-to-batch reproducibility of scaffold chemistry is essential to establish correlation between scaffold features and cell responses. Our experience on *in vitro* 3D bone model shows that a basic CaP ceramic scaffold with optimized architecture can achieve the

right permeability, bioactivity and mechanical properties to seed, grow and mechanically stimulate primary cells in a reproducible manner (109).

2.5. Future Challenges and strategy

As already stated, robust spatial and temporal control of biophysical, biomechanical and biochemical micro-environmental cues through 3D biomaterial-based culture systems is essential for studying cell regulatory pathways and *in vitro* tissue regeneration. In this context, experimental models will have to increase progressively their complexity from basic to physiologically relevant structural and functional features (e.g dynamic and complex microenvironment of the native stem cell niche). A core challenge of this strategy will be to uncover the relationship between chemical (e.g. degradation), physical (e.g. microporosity) and mechanical (e.g. stiffness) scaffold properties, and cell signaling (32). This implies the accurate control and characterization of architectural, mechanical and physico-chemical biomaterial features, at micro- and macroscales, from synthesis, through shaping, until the sterilization steps. Unfortunately, physico-chemical properties (for review (104)) as well as architectural features (58) of scaffolds are still too often considered as non-significant parameters and thus become confounding variables; as evidenced by Kumar et al. speaking about the “unexpected sensitivity that cells have for scaffold structure” (67). In most cases, the whole scaffold manufacturing process (e.g. chemicals sourcing, phase synthesis, scaffolding method) is not enough accurate/standardized, and the final product not enough characterized to elucidate the link between scaffold features and biological response (38, 110, 111). For instance in another study of Kumar et al., is the osteogenic differentiation of hBMSCs, seeded on etched poly(ϵ -caprolactone) scaffolds, induced by surface roughness or surface chemistry change (112) ? It is also unfortunate that series of studies on *in vivo* and *in*

vitro evaluation of silicon-substituted hydroxyapatite ceramics would have been published without any information about the real phase composition of the samples (110, 113), when it was recently shown that even small changes of silicon-substituted hydroxyapatite ceramic phase composition drastically modify cellular responses (114, 115).

Defining the appropriate(s) composition(s) and the relevant architecture(s) of these new 3D systems for specific experimental goals remains a significant challenge (32). However, new design concepts and fabrication techniques currently available (e.g. additive manufacturing), even if they still display inherent feature limitations (81), provide alternatives for developing scaffolds with sufficient batch-to-batch similarity in geometrical, mechanical and physico-chemical features to enhance our understanding of cell signaling (82, 83). To reach this goal both computational simulations and experimental studies are necessary, including extensive characterization of main scaffold features before and during *in vitro* tests in order to refine the numerical models.

Therefore, we propose that, as an initial step, scaffold design should follow a simple -even simplistic- approach, comprising:

(i) an isotropic macroporous architecture with continuous open pore geometry such as the one based on triply periodic minimal surfaces (TPMS) (50, 74). Compared to a random-pore architecture, this “basic” design could simplify both the identification of the variables affecting cellular response (by limiting assay variability and standardizing lab to lab assays), and the numerical simulations of the different physical variables which characterize the 3D biomaterial-based culture system (e.g. fluid flow profile experienced by cells). This architecture could also facilitate a dynamic monitoring of the cells by non-invasive imaging, for instance through confocal microscopy (50, 83).

(ii) a monophasic material with homogeneous and reproducible surface properties. The production of such material implies a perfect control of all production steps from chemical synthesis to sterilization.

(iii) an accurate characterization of the actual chemical, physical, mechanical, architectural and biological properties of the manufactured scaffold by means of experimental techniques (e.g. μ CT, spectroscopy, mechanical tests, osteoclast culture, degradation experiment), and computational methods (e.g. computer fluid dynamics simulation, finite element analysis). Since the properties of the scaffold are not only affected by cell activity, but also by culture conditions (e.g. mechanical stimulation, fluid flow, media composition), their evolution should be quantified in these conditions, i.e. without cell.

3. CELL CULTURE

3.1. Cell types and sources

In bone biology, osteoblast-like cells are the most studied because they are easier to obtain, cultivate and use compared to osteoclast-like cells or osteocytes. For a comprehensive review focused on osteoclast-like cells or osteocytes, see (116) (these cells will not be discussed herein). Cells used for *in vitro* bone studies are either cell lines or primary cells isolated from human or animal tissue. In both cases, these cells display more or less differentiated features, ranging from stem-like cells to osteoblasts. Consequently, the choice of the cell type model (cell line or primary cells? Which cell lines? etc.) will depend on the goal of the study.

A cell line is a homogeneous population of cells with the ability to proliferate in culture without limit, at least in theory, and therefore called “immortal” (117, 118). Cell lines are

obtained from cells isolated from bone tumors (cancer cell line) (119) or from primary bone cells which have undergone a spontaneous genetic change, such as p53 mutation (non-transformed cell lines) (120). Primary cell can also be genetically/artificially modified to be "immortalised", they are then named "transformed cell lines" (121). The advantage of cell lines is the immediate availability of a large numbers of cells without the need for isolation, the ease of maintenance and the homogeneity of the cell cultures. Cell lines are less prone to the phenotypic variability observed in primary cell cultures, but at the cost of some functional characteristics that are lost after immortalisation. Thus, cell lines may not express all tissue-specific characteristics.

For example, the non-transformed murine preosteoblasts MC3T3-E1 cell line (122) is commonly used for its high capacity of differentiation in response to materials (apatite), with or without differentiation medium (123). However, cultures of these cells have been shown to not mineralize as hydroxyapatite but in spots of a mineral of unknown form (124). Human osteosarcoma cell lines, such as MG-63 and SaOS-2, particularly useful in the study of interactions between osteoblasts and materials (125), as well as assessment of biocompatibility and osteoblast differentiation potential (126, 127), must also be used with caution. Indeed, the response obtained with these lines may differ from those obtained from healthy cells. As a case in point, Anselme team compared the deformation of the cell nuclei in response to substrate surface topography in "healthy" (HOP), cancerous (from osteosarcoma: OHS4, U2OS) and immortalised (F/STRO-1+A and FHSO6) (bone-forming) cells (128). Cancer cells displayed a strong deformation, immortalised cells showed reduced deformation, while healthy cells were deformed only after short incubation times. Along with other results (129, 130), this study highlights the impact of immortalization on the state/responsiveness of the cytoskeletal network, leading to a modified behaviour compare

to healthy cells (128). Overall (see also (116, 131)), cell lines are useful for specific studies such as cytocompatibility testing or various stages of osteogenesis investigation, but do not reflect the behaviour of primary cells. Additionally, many cell lines can become unstable with time, and subclones of these cell lines tend to develop in different laboratories, which can lead to different experimental outcomes and results no longer comparable between studies and/or laboratories.

Primary cells provide an alternative to cell lines. They are “unadulterated/healthy” cells, cultivated directly from human or animal tissue. Populations of bone primary cells contain stem cells and osteoprogenitors (i.e. committed but undifferentiated cells). An evident advantage of primary cells is that they can be isolated from genetically modified animals in which the expression of a gene has been cancelled, forced or rendered conditional, as for instance BSP-/- mice used in our laboratory (132, 133). This avoids the direct manipulation of gene expression in the culture (e.g. RNA silencing), for which the transfection of the agent, as well as the efficiency and duration of its action are difficult to control.

Many methods are available for the isolation of primary cells from bone. A common one is to digest small pieces of bone explants from long bone, calvaria (from rodent fetus or neonate), mandible or iliac crest with collagenase or other matrix-digesting enzymes (134, 135). Primary cells are also regularly isolated from the bone marrow (136).

Bone marrow in adults harbours several types of stem and progenitor cells, hematopoietic (HSC) and non-hematopoietic, the latter including mesenchymal stem cells (MSC). MSC are undifferentiated cells with high proliferation capacity, able to self-renew and differentiate into different cell lineages, generating all mesenchymal tissues, such as bone, cartilage, tendons, muscles and fat (137). *In vitro* differentiation studies demonstrated the ability to isolate and multiply MSC from cartilage, trabecular bone, periosteum, adipose tissue and

periodontal ligaments (138-142). However, gene and protein profiles characteristic of these cells are still unclear. Recently, the European Genostem consortium elucidated the biology of stem cells from bone marrow, establishing that the native cells are localised on the abluminal side of endothelial cells of sinuses (143). They showed that stromal cells forming the niche of HSC and bone marrow MSC are the same entities, and that highly proliferating clonal cells, amplified in culture, are "true" stem cells since they display self-renewal and multipotency, similar to HSC (143). However, many questions remain unresolved. It is not clear, for instance, whether the self-renewal capacity of MSC is comparable to that of HSC (143). Thus, several important players in this field consider that the only true test of stemness is *in vivo* tissue/organ reconstitution from one single MSC, and they contest the notion of a universal MSC as opposed to tissue-specific (e.g. in the bone marrow, skeletal) stem cells (144).

Anyway, the main practical disadvantage of the use of primary cells is that considerable variation may exist between cells of different tissue sources. Cell populations derived from a single tissue source may also be heterogeneous due to variations in the isolation and/or purification technique, or according to passage numbers. For instance, significant changes in contractile force were reported for chondrocyte after only three passages (145). In addition, primary cells are extremely sensitive to the batch of serum used (146-148), emphasizing the importance of the culture medium composition. The addition of certain factors, such as dexamethasone, ascorbic acid and β -glycerophosphate allows to direct these cells towards the osteoblastic differentiation pathway. Similarly, other inducing media exists to drive differentiation to adipocytes or chondrocytes. Also, in order to improve the homogeneity of marrow-derived populations, it is possible to sort and select cells with antibodies targeting cell type-specific membrane markers (149).

3.2. Complex 3D models: cell co-culture

Tissue engineering mainly uses "minimalist" approaches using a single cell type. In contrast *in vivo*, the dynamics of the bone tissue – in particular, its remodelling - is based on an intimate cross-talk between different cell types: endothelial cells, matrix-embedded osteocytes, bone lining cells and more particularly osteoblasts and osteoclasts (150).

In vitro osteoclast/osteoblast co-cultures on scaffolds allow the study of the remodeling process and, in particular, the crosstalk between resident osteoblasts and invading, differentiating, and resorbing osteoclasts. Tortelli et al. (151) developed a 3D model of bone by seeding primary murine osteoblasts and osteoclast precursors in a ceramic porous scaffold (placed in multiwell plate). Using this model, the team demonstrated through comparative analysis of transcriptional activities that the 3D environment stimulates osteoblast differentiation which, in turn, induces early differentiation of osteoclasts. Vacanti team also highlighted, in an osteoblast and osteoclast co-culture experiment, the formation of osteoclasts on polymer surfaces previously mineralized by osteoblasts Nakagawa (152).

Osteoblasts and osteoclasts are not the only critical cells for bone remodeling. For a very long time, osteocytes have been ignored because it was assumed that they functioned as place holders in the bone matrix and that all the action took place on the bone surface. However, within the last decade these cells have been shown to act as orchestrators of bone remodeling (153, 154). More precisely, several experiments using conditioned medium (i.e. supernatant containing soluble factors from osteocytes) (155, 156) or different co-culture methodologies (155, 157, 158), in 2D substrate or in 3D gels (158, 159), support the concept that osteocytes regulate the function of both osteoblasts and osteoclasts. Moreover,

considerable convincing data emphasize the role of osteocytes as mechanosensors, integrating mechanical signals to regulate bone mass (153). Indeed, in experiments mechanically using parallel plate flow chambers, mechanically stimulated osteocytes inhibit osteoclastogenesis (160) and increase osteoblastic activity (laminar flow model, (161)).

In vivo, angiogenesis and osteogenesis are closely linked and stringently regulated. Thus, interactions between osteoblasts and endothelial cells have been extensively studied. Complex cellular crosstalk between these cells have been reported in several *in vitro* studies (162, 163) using different co-culture models. Grellier et al. (164) made an inventory of these models, from the nature of the 2D and 3D substrates (e.g. spheroid or porous scaffolds with different compositions), to the cellular models used (e.g. rat long bone-derived osteoblast-like cells, human umbilical vein endothelial cells or human bone marrow stromal cells) and the crosstalk between these different lineages. Co-culture of either osteoblasts or osteoprogenitor cells with vascular cells led to opposing conclusions about the ability of endothelial cells to induce or inhibit the expression of osteogenic genes (165). Indeed, such interactions depend on the origin and differentiation state of the two cell types, as well as the co-culture system used (2D or 3D substrates). However, when endothelial cells had an inducing effect, the *in vitro* concomitant mineralization of deposited matrix and formation of blood vessels have not been observed. Thus, there is much more to learn about the chemical and physical signals that drive the coordination of angiogenesis and osteogenesis *in vitro*.

Recently, Papadimitropoulos et al. (166) developed a more complex *in vitro* model to mimic bone turnover by co-culturing osteoblasts, osteoclast and endothelial cells. To address the issue of the very short life of osteoclast cells, the authors developed a culture process in two steps. First, cells from the stromal-vascular fraction of adipose tissue, containing both

osteoprogenitors and endothelial lineage cells, were seeded then cultivated for a week in a perfusion bioreactor. In the second phase, CD14+ human peripheral blood cells were infused into the scaffold via the perfusion system. Non-invasive monitoring techniques (e.g. analysis of typical markers of bone remodeling in the culture medium) were used to quantify the bone matrix deposition and resorption processes, as well as to document the functional regulation of the system via osteoclastogenic and osteotropic factors (166). Unfortunately, these results do not allow to fully characterize the “quality” of the bone-tissue like formed (167). Bone quality encompasses several parameters such as bone remodeling level, matrix composition, bone mineralization degree, matrix and mineral composition. Monitoring of all these parameters are still missing in *in vitro* studies, which therefore do not established that the “bone-tissue like” material formed in the culture is comparable to “genuine bone”.

Nonetheless, all co-culture studies, regardless the cell types used are useful to better understand the crosstalks regulating bone physiology. Most try to come closer to the physiological environment of the cells by using 3D matrices or mechanical stimulation. However, no study combines these two important conditions of the cell environment. It is thus a major issue to co-cultivate cells in 3D models allowing different type of mechanical stimulations.

3.3. Cell seeding

The cellular seeding of a porous scaffold is an important step in the setting up of a 3D culture. First, the initial seeding density impacts tissue formation. High cell densities are associated with stimulation of osteogenesis and increased bone mineralisation (168). Classically, a 30 mm³ macroporous biomaterial is inoculated with one million cells (169, 170). Second, the seeding protocol also affects the viability and distribution of cells within the scaffold, impacting the subsequent distribution of the tissue formed (171). Uniform cell

seeding is thus necessary to get uniform cell response within the scaffold and reproducible 3D cell culture.

3D cell seeding is generally performed under static conditions by placing a suspension of cells on top of the scaffold, which will penetrate within the material by capillary and gravity action. Although this standard method is widely used, it may result in low seeding efficiency and non-homogeneous distribution of cells in the scaffold (171-173). The seeding efficiency can be improved by placing the scaffolds in a stirred cell suspension. Spinner flask systems have been used for this purpose. However, the inefficient convection of the interior of the scaffolds can also lead to low cell densities and non-homogeneous distribution of cells (171, 174) with a higher density at the external surfaces (175). To further improve seeding efficiency, a flow of cells in suspension can be forced through the scaffold by means of a perfusion bioreactor. In this case, seeding and cell growth are successively performed without further manipulation, greatly reducing the risk of contamination. The use of perfusion bioreactor for cell seeding, developed in the next part of this review, allows a more homogeneous distribution of cells within the scaffold compared to static seeding or with spinner flask (171-173). However, the effective seeding conditions (e.g. seeding flow) have to be adjusted according to the type of perfusion bioreactor, the scaffold internal architecture, as well as the cell type(s) used.

4. PERFUSION BIOREACTORS: the solution for *in vitro* 3D bone model(s)

Nowadays, it is well established that a perfusion-based bioreactor is the most efficient culture system to homogenise nutrient transport, waste elimination as well as cell distribution, growth and differentiation in the interior of the scaffold (4, 176). These systems consist in forcing the culture medium through the cell-seeded scaffold. They are usually

composed of a scaffold chamber with a reservoir and a pump, all connected by tubing (**Fig. 2 A**). Many perfusion bioreactors have been designed according to the goal of the study, using various scaffolds, cell types and perfusion modalities (i.e. mode and rate of fluid flow). These systems have been detailed in several interesting reviews (4, 5, 177). In addition to control and monitor many culture parameters throughout the tissue growth *in vitro* (e.g. pH, temperature, pressure, nutrient supply, waste elimination), perfusion bioreactors allow to study the role of mechanical cues on cell fate. Indeed, the perfusion and/or loading systems combined with the bioreactor can be used to mechanically stimulate the cells.

4.1. The media: controlling and monitoring

In order to live, a cell needs nutrients such as glucose, oxygen, amino acids, fatty acids, vitamins as well as specific factors. Among them, oxygen is much more than a required nutrient, it can act as signalling molecule to affect cell behaviour in several ways (178). Thus, high oxygen concentrations appear toxic to many cells, while low levels of oxygen can promote cell viability (179) and the differentiation of stem cells *in vitro* and *in vivo* (180-183). Moreover, while it is clear that the local oxygen pressure is a potent regulator of metabolism and viability of bone cells, the distribution profile of oxygen in bone is currently unknown. Nonetheless, mathematical models suggest that significant oxygen gradients exist between the cortical and cancellous bone, and that the partial pressure of oxygen (PaO_2) can regulate the physical dimensions of trabeculae and osteons (184). Moreover, it is known that *in vivo*, the PaO_2 is 5% in peripheral tissues, 6–7% in bone marrow and 1% in the endosteal niche (183). It should be noted that these values are low compared to the 21% PaO_2 usually used in classical (2D) cell culture conditions. Apart from oxygen, a recent study showed that glucose concentration is an important factor in maintaining the viability and growth of MSC

in vitro (185). Deschepper et al. showed that MSC could sustain near-anoxia conditions only in the presence of glucose *in vitro* and *in vivo* (186).

Thus, culture conditions and especially the chemical composition and homogeneity of the medium influence cell viability and behaviour. In static conditions, pure diffusive transport ensures oxygen and nutrients supply to the interior of the scaffold only to a distance of about 100 μm from the scaffold outer surface (187), similar to a 2D culture. In these conditions, cells are subjected to different oxygen and nutrient concentrations across the scaffold, leading to the formation by the outer cell layer of a tissue capsule in the periphery of the scaffold, further limiting penetration of culture medium inside the construct (176, 188). More generally, the composition of the culture medium, including the availability of nutrients and the removal of waste products (e.g. CO_2 , lactic acid, ammonia and products generated by oxidative stress), as well as the rate of exchange between the bulk medium and the construct interior are key parameters for 3D cell culture *in vitro* (4). Both parameters depend on the scaffold mass transfer properties (internal pore architecture discussed in section 2.3) and the culturing schemes (e.g. static vs perfused). As stated in introduction to this section, the perfusion bioreactor systems are currently unrivalled in terms of performance (e.g. cells activity and uniformity within the interior of the scaffold (176, 189, 190). On the condition that the flow goes entirely through the scaffold and not around the edges, these systems allows an accurate control of the perfusion modalities of seeded cells (e.g. constant vs pulsatile) as well as their mechanical stimulation (see section 4.2), with the final aim to modulate stem cell fate (maintenance or differentiation) (4, 178).

Finally, another advantage of perfusion bioreactor is the monitoring of crucial parameters like oxygen using invasive (embedded) or non-invasive (non-contact) sensing probes (191).

These monitoring tools/probes can also be used to follow the bone remodelling process as performed by Papadimitropoulos et al (see section 3.2) (166).

4.2. Control of the mechanical stresses

4.2.1. Mechanical bone environment

In vivo, it is well known that bone remodelling is induced and controlled by endogenous and exogenous factors (192-194). Dynamic mechanical stresses are osteogenic within a range of load intensities; beyond and below this range tissue responses are different (195, 196).

In weight bearing bone, individual cells are not subjected to a single force. Multiple physical signals are generated simultaneously and sensed/integrated by the cells through their membrane, cytoskeleton, focal points, etc. (**Fig. 3**). Consequently, there is no single parameter in the mechanical environment that reliably predicts bone remodelling *in vivo* or under experimental conditions (197).

It is assumed that *in vivo* mechanical stimuli are mainly transmitted to bone cells through shear stress, that is to say forces whose vector is parallel to the cell surface (198). Bone tissue includes a significant fraction of interstitial fluid surrounding all cells. Movements of this fluid are induced by two different processes: an "internal" one, which is the differential pressure within the circulatory system (199, 200), and an "external" one consisting in the mechanical loads applied to the skeleton (201). Whenever a mechanical load is applied to bone, interstitial fluid is forced out of the areas of high compressive deformation, and flows back when the load is removed. Thus, the fluid flow generates an oscillating shear stress to which individual bone cells, and more especially the osteocytes, are sensitive (154).

The different forces exerted on cells are integrated via several extracellular mechanosensor like integrins and associated proteins (203), ion channels (204), connexin (205), primary cilia

(206) and the actin cytoskeleton (207). These sensors take part in a set of cellular mechanisms that convert mechanical stimuli into biochemical signals (208) leading in changes of whole tissue metabolism (**Fig. 3**).

In 3D models, many mechanical stresses can be generated and studied such as tension, torsion and different combinations of these. In fundamental bone *in vitro* models, two types of mechanical stresses are mostly studied: loading stress resulting from mechanical solicitation, and shear stress imposed by fluid movement from the perfusion and/or the scaffold deformation (27).

Anyway, the control of these stresses is crucial to understand the transduction of mechanical signals by the bone cells or guide their fate.

4.2.2. Fluid flow effects

a) Shear stress in perfusion bioreactor

In addition to overcoming poor nutrient and waste exchange associated with static culture conditions (188, 209), culture in a flow perfusion bioreactor provides a convenient way to mechanically stimulate cells seeded in the scaffold through fluid shear stress (198, 210). Indeed, it was proved in numerous 2D *in vitro* studies that cells involved in bone tissue remodeling are sensitive to shear stress (211-213). In scaffolds, fluid flow (shear stress) has been also shown to enhance bone formation activity of osteoblasts (214-216) and stimulate the expression of bone-specific genes (217). An unidirectional steady flow, commonly used in clinical studies (214, 215, 218), has been shown to have many biological effects on bone marrow stromal cells seeded in scaffolds, such as an enhancement of osteogenic differentiation (219) and an increased production of mineralized matrix (214, 220). However, since bone cells are mainly stimulated *in vivo* via dynamic (intermittent) mechanical loading

(221, 222), this dynamic regime is likely to be crucial for stimulating bone formation and enhancing the quality of bone tissue laid by osteoblast (223). Other studies revealed a favorable effect on bone tissue formation of dynamic unidirectional flow (224) or oscillatory fluid flow (170, 225). Lastly, a recent study showed that sequential application of steady (for 2 weeks) and dynamic (for 3 weeks) fluid flow promotes bone formation by human adipose stem cells isolated from fat tissue and cultivated on porous silk fibroin scaffolds (226).

These results suggest that there is a cellular mechanism detecting and responding to the level and duration of shear stress induced by fluid flow. The level of shear stress experienced by cells cultured in a flow perfusion system varies with the flow rate. The local shear stress felt by individual cells will thus depend on the flow rate used throughout the perfusion-seeding (172, 227), as well as the scaffold architecture (see section 2.3). It remains unclear which flow profile is optimal for stimulating osteoblasts cultivated in a scaffold, and how it will regulate their activity and sensitivity. Such evaluation can be done using predictive tools (computational fluid dynamics technology (228, 229)).

b) Biologically relevant shear stress

Most culture systems and bioreactors are not yet optimized on a mechanical point of view (mechanical stimuli generation). In the design of the perfusion bioreactors (3D *in vitro* studies), parameters, such as rate of perfusion, flow design (unidirectional, oscillatory, intermittent, etc.) and scaffold architecture have often been determined through trial-and-error (54, 230). Such empirical methods do not guarantee that these parameters are optimized. But computational tools are now available to determine the optimal values of these parameters. In order to improve the mechanostimulation of cells in bioreactors, it is

first necessary to determine which levels of shear stress are relevant, and secondly to be able to evaluate the shear stress levels on scaffolds surfaces.

- Effective levels of shear stress in 2D cultures

It is relevant to wonder about the levels of shear stress that provide the best stimulation in bone cell function. A first value of reference was given by Weinbaum et al. in 1994 (231) through a model based on Biot's porous media theory, which is used to relate the loads applied to bone to the flow of canalicular interstitial fluid. The model predicts that the flow of physiological fluid induces shear stresses in the range of 0.8 to 3 Pa when bones are submitted to physiological loadings.

Many research teams analyzed experimentally the effect of shear stress on bone cell activity. To control the shear stress applied, parallel-plate flow chambers were developed and used *in vitro* with several types of cells and several shear stress regimens (e.g. frequency, time). A selection of the most relevant results is presented in **Table 1**. In these 2D experiments, shear stress levels between 0.1 and 2 Pa caused positive effects on cells such as release of nitric oxide and PGE2, phosphorylation of proteins ERK1/2 and p38, as well as up-regulation of the expression of some genes related to mechanotransduction and osteoblastic differentiation.

- Levels of shear stress in 3D, numerical simulation and confrontation with 2D values

Scaffolds used for bone tissue engineering applications provide, by definition, a 3D structure with generally complex geometries. It is then impossible to control and homogenise the levels of shear stress experienced by cells in such structure. But some numerical methods were developed to evaluate these levels. Nowadays, numerical simulation is very widely used by researchers to simulate mechanical and/or biological environment experienced by cells seeded in scaffolds, to become a crucial research field. For an extensive review of

current simulation techniques and strategies for dynamic bone tissue engineering in bioreactors we recommend a recent review by Vetsch et al. (239). Several research teams investigated the levels of flow-induced shear stress in their own system (i.e. bioreactor and scaffold (240-242)). A first step of modeling is generally achieved by analyzing the scaffold through micro-computed tomography in order to reconstruct its 3D architecture numerically. The reconstructed geometry of the scaffold can then be implemented in numerical simulations. Computational fluid dynamics methods for fluid simulation are used, such as Lattice Boltzmann (240, 243), finite volume (70, 241, 244) or finite element methods (71, 245, 246). Boundary conditions are implemented to reproduce the real fluid circulation. Then, calculation enables to determine the fluid velocity and the wall shear stress in the scaffolds. The shear stress levels calculated under certain hypotheses are listed in **Table 2**. We would like to emphasize that due to the highly randomized internal pore structure of the scaffolds used for these experiments, the shear stress distribution is very heterogeneous and we should retain the order of magnitude rather than the values themselves. It is very interesting to note that the calculated shear stress levels are in the range of 0.04 to 40 mPa with boundary conditions of the numerical models representing the experimental conditions. These values are two to three orders of magnitude lower than the values reported in the 2D experimental studies (**Table 1**). This gap has been reported and explained in several works (247, 248). When cells are seeded in highly porous scaffolds, approximately 75% of them display a “bridging” morphology (i.e. they are suspended cells, as observed in **Fig. 1 C**), in contrast to the “flat” 2D morphology (i.e. in which cells lay flat on the substrate) (247). Even if this proportion could vary depending on internal pore architecture and cell type, these “bridging” cells exist and experience high levels of deformation compared to the “flat” cells; up to 500 times higher for the same flow configuration. This is very probably why

the osteogenic responses can be observed at lower levels of wall shear stress in scaffold structures containing mainly bridging cells than in 2D culture experiments or in other types of bioreactors where cells are mainly flatly attached.

4.2.3. Effects of mechanical loading

While shear stress has been extensively used to mechanically stimulate cells in 3D, little has been done on cell response induced by compression forces *in vitro*.

Specially designed culture chambers are necessary to apply external mechanical signals combining shear and loading stresses (64, 109, 249, 250) (**Fig. 2 A**). This type of chamber makes possible to mimic *in vitro* the skeletal effects of physical activity. Mechanical loads generate substrate deformations inducing in parallel fluid flow shear stress (media movement within the scaffold). Both are more or less intense depending on the properties of the scaffold (see section 2). These deformations are experienced by the cells, through the ECM or directly through changes in cell shape or intercellular distances. Substrate elongation or compression can directly modify extracellular gradients, cell-cell communication or local concentrations of secreted ligands (251).

Globally, bioreactor systems designed for applying mechanical loading have shown beneficial effects of mechanical load on proliferation, osteogenic differentiation and matrix formation (**Table 3**). Specifically, it has been shown that compressive strains of approximately 10% (amounting to a stress of about 12 kPa) are osteogenic (**Table 3**). Other bioreactors (prototypes) have been designed and technically validated but have not been yet used in routine for biological evaluations (255, 256). Some of the bioreactors listed in **Table 3** are not perfused. This technical restriction limits their experimental applications due first to the negative points inherent in the nature of the unperfused systems and especially static

cultures (see section 4.1), and second to the impossibility to combine the effects of both compression and perfusion.

Lastly, all these studies are currently done with osteoblast-like cells in order to determine the effect of mechanical forces on bone formation. The next step will be to study their effects on bone resorption using (pre)osteoclasts with the ultimate goal to co-cultivate either or both cell types with the bone mechanosensor, the osteocyte.

5. PERSPECTIVES IN 3D IMAGING

As the field of 3D tissue engineering progresses there is a growing need for non-invasive, label-free imaging technologies for detailed analysis of the inside of the scaffolds, with high resolution and real-time observation capabilities throughout the culture period. Two on-line imaging systems able to monitor the 3D morphological structure of the mineralized and unmineralized fractions of the cultures will be presented: Optical Coherence Tomography (OCT) and the micro/nano tomography.

Operating under a principle similar to ultrasound imaging, with the exception that it measures delays in backscattered light instead of sound, OCT uses light in the near infrared part of the spectrum. OCT yields 3D tomographic images of biological tissues with a resolution of a few micrometres. This non-invasive, non-ionising method has been recently used for imaging a fixed human femoral trabecular bone sample (257). Without any staining, the lamellar structure of trabeculae and the cells in the marrow were visible, albeit with a penetration depth limited approximately to 1 mm (258, 259). To improve the capabilities of OCT several extensions have been proposed including polarization, phase and spectrally resolved OCT. Spectroscopic Optical Coherence Tomography (SOCT) is sensitive to both chromophores and structural changes of the tissue, which alter the incident light spectrum.

The performance of such approach has been tested for contrast enhancement in bovine osteo-articular cartilage. Specifically, when the tissue is loaded the different layers of cartilage get enhanced contrast due to optical clearing. During cartilage compression, water is expelled out of the tissue and the change of the collagen fibre composition in the different zones could be a reasonable explanation for the enhanced contrast due to spectroscopic features (260). Whether some interesting results on structural investigation of native bone have been gathered, the applications of these techniques to *in vitro* tissue-engineered bone are not yet very numerous at present (260).

Micro-computed tomography (micro-CT) is often used to characterise scaffolds and bone growth in 3D (261). Micro-CT uses X-rays and provides a non-destructive 3D image of the interior of a sample, regardless of size, with a resolution ranging from 1 to 100 μm . The combination of micro-CT and recently developed microdiffraction, x-ray techniques have facilitated qualitative and quantitative characterisation of dense scaffolds, as well as a 3D evaluation of the rate of bone growth in engineered tissue (262). More specifically X-ray microdiffraction provides information at the atomic scale, i.e. at the nanometre level, of both mineral and organic phases. Wide angle X-ray scattering (WAXS) informs mainly about the crystallographic structure and texture of the samples, while Small angle X-ray scattering (SAXS) is more about size, shape and orientation of both the mineral crystals and collagen fibrils (262) which compose the sample. Synchrotron radiation tomography (SRT) has enabled acquisition of even better results in terms of spatial resolution (under 1 μm) and signal/noise ratio (263). In addition to revealing the architecture of the sample, SRT also provides information about composition (i.e. the 3D distribution and degree of mineralisation) (264). However, equipment to perform this technique is only available in a few research centres around the world.

Micro-CT and SRT techniques allow visualisation and quantification of mineralised structures, including the possibility to investigate the influence of scaffold features, such as the internal pore architecture (e.g. size, distribution) with regard to the growth of bone within the scaffold. Unfortunately, this method does not yet allow to visualise the cells. "X-ray synchrotron radiation pseudo-holotomography" was recently developed to visualise soft tissue and microvascular networks in 3D without the use of contrasting agents. The method is based on micro-CT and conventional holotomography techniques and was used to obtain quantitative structural data on a bioceramic/MSC composite implanted in mice for 24 weeks (265).

If major improvements in mapping and monitoring 3D tissue growth have been achieved, progress is still required in quantifying changes in depth with the appropriate resolution for cellular analysis. Further bioreactor should be designed to allow for in situ imaging with the help of optical and/or x-ray transparent culture chambers or by placing transparent windows on the chamber walls.

6. CONCLUSIONS

Traditional cell culture, i.e. two-dimensional (2D), is unable to reproduce the tissue properties observed in organs (3D). Moreover, natural tissues, especially in weight-bearing organs such as bone, are subjected to mechanical stresses which are major regulating factors for cell fate. *In vitro* 3D culture models seem to be an effective solution to better understand the different aspects of cell function and bone remodeling in systems less complex than *in vivo* models. Create bone *in vitro*, using tissue engineering concepts, can be a somewhat elusive vision given the complexity of this tissue, encompassing spatial and temporal interactions between many types of components (e.g. cells, ECM, substrate, soluble factors).

However, new experimental, manufacturing, characterization and computational tools help to design a controlled environment more and more efficient to regulate cell fate. Even, if the influence of material properties, such as stiffness, permeability, or composition, in orchestrating cellular events is still poorly understood, the “design strategy” associated to an extensive scaffold characterization is a consistent approach to define relevant scaffold design/features. Perfusion based bioreactors have been shown to optimize cell culture in 3D with full and standardized control of cell environment (e.g. scaffold architecture and physico-chemical properties, medium composition and delivery, mechanical stimuli) (**Fig. 4**). This new way to cultivate cells requires a redefinition of standard cell culture methods applied in two dimensions (2D) starting with cell seeding techniques and growth conditions (e.g. O₂ levels). The development and follow-up of such controlled and constrained systems also calls for a multidisciplinary knowledge, relying on close collaboration between researchers and engineers specialising in biology (molecular and cellular), chemistry, physics and mechanics (of solid and fluid matter).

Development of advanced methods for real-time analysis is required to characterize and monitor the physical and chemical environment of the cells and their interactions with the construct. No 3D *in vitro* culture strategy can provide a "complete" model of bone tissue (i.e. its different components). For this reason, rather than attempting to build the perfect general 3D bone model, research should focus on developing specific tools for the purpose of solving one or several well thought biological questions. To conclude, this approach will serve basic research, provide a test bench for the development of active molecules targeting bone cell as well as therapeutic applications (implantations). The trial-and-error approach mostly used to validate scaffold/biomaterial properties for implantation (e.g. composition,

architecture) or implant manufacturing (e.g. rhBMP2 dose, preculture in bioreactor before implantation) could be guided by results obtained in these *in vitro* 3D culture systems.

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Tables

Table 1: Effects of shear stresses on bone cells seeded on glass in parallel plate flow chambers

| <i>References</i> | <i>Cell types</i> | <i>Shear stress level</i> | <i>Shear stress regimen</i> | <i>Observations</i> |
|-------------------------|---|---------------------------|--|---|
| Bakker, 2001 (232) | Mouse primary cells from adult long bone | 0.4 Pa, 0.6 Pa and 1.2 Pa | Pulsating fluid flow, 3Hz, 5Hz or 9 Hz, 15min | NO and PGE ₂ production |
| Alford, 2003 (233) | Mouse osteocyte-like cell line, MLOY-4 | 1 Pa | Oscillating fluid flow, 1Hz, 1h | Phosphorylation of Cx43 Up-regulation of GJIC |
| Kapur, 2003 (234) | Human primary osteoblasts from mandible bone chips | 2 Pa | Steady fluid flow, 30min | Increase of [³ H]thymidine incorporation, ALP activity, phosphorylation of ERK and expression of integrin β 1 |
| Bacabac, 2004 (235) | Mouse pre-osteoblast cell line, MC3T3-E1 | 0.7 Pa | Steady and pulsating fluid flow, 5 or 9Hz, 15min | NO production (NO ₂ ⁻ accumulated in the conditionel medium) |
| Knippenberg, 2005 (236) | Goat primary mesenchymal stem cells from adipose tissue | 0.6 Pa | Pulsating fluid flow, 5Hz, 1h | Increase of NO production and upregulation of Cox2 gene expression |
| Kreke, 2005 (237) | Rat primary bone marrow stromal cell culture | 0.16 Pa | Steady fluid flow, 5min, 30min or 120min | Increase of ALP activity, increase of the expression of osteopontin and bone sialoprotein mRNA, increase of PGE ₂ |
| Mullender, 2006 (238) | Mouse pre-osteoblast cell line, MC3T3-E1 | 0.18 and 0.64 Pa | Steady and pulsating fluid flow, 1Hz, 5Hz or 9Hz, 15min | NO and PGE ₂ production increased |
| Kreke, 2008 (211) | Rat primary bone marrow stromal cell culture | 0.23 Pa | Continuous and intermittent (5min on/5min off) fluid flow, 24h | Increase of the phosphorylation of ERK and p38 Increase of PGE ₂ |
| Grellier, 2009 (212) | Human primary bone marrow stromal cell culture | 1.2 Pa | Steady fluid flow, 30min and 90min | Gene expression : ALP increased, Cx43 increased, Col I decreased Phosphorylation of ERK1/2 and p38 |

Table 2: Numerical models prediction of shear stress values induced by flow within scaffolds

| <i>References</i> | <i>Scaffold</i> | <i>Numerical method</i> | <i>Calculated mean wall shear stress</i> |
|-----------------------|---|-------------------------|--|
| Porter, 2005 (240) | Human trabecular bone | Lattice-Boltzmann | 0.05 mPa |
| Boschetti, 2006 (245) | Polylactic acid (PLLA) porous scaffold | Finite element method | 3.7mPa (« Reference model ») |
| Cioffi, 2006 (241) | Biodegradable polyesterurethane foam scaffold | Finite volume method | 3.94, 3.73, and 3.28 mPa vs model |
| Cioffi, 2008 (70) | Polyethylene glycol terephthalate/polybutylene terephthalate (PEGT/PBT) foam scaffold | Finite volume method | 2.56 and 0.256 mPa vs inlet flow |
| Sandino, 2008 (308) | Porous and biodegradable scaffolds : calcium phosphate and glass | Finite element method | 0.05 mPa (Modal values) |
| Milan, 2009 (71) | PLLA-glass scaffold | Finite element method | Between 0.04mPa and 40mPa vs inlet flow |
| Melchels, 2011 (50) | 2 PLLA scaffolds with gyroid pore architectures | Finite volume method | 31 and 27 mPa |
| Yan 2011, (229) | Chitosan-hydroxyapatite scaffolds with regular structure | Finite element method | Between 1 and 7 mPa |
| VanGordon, 2011 (243) | PLLA porous foam scaffold and nonwoven fiber mesh scaffold | Lattice-Boltzmann | Between 12 mPa and 25 mPa vs inlet flow and geometry |
| Maes, 2012 (244) | Titanium and hydroxyapatite scaffolds | Finite volume method | 1.41 and 1.09 mPa |

Table 3: Direct mechanical loading of cells seeded scaffolds – Modalities and effects

Bouet.Table3

| Scaffolds | | | | | Cell types | Effects |
|--|---|---|--|--|---------------------------------|---|
| Composition | Porosity | Perfusion modalities | Compression modalities | | | |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | Interconnected macropores | 0.1 mL/min (peristaltic pump) Constant unidirectional | 1Hz ~1.5% strain 1h/day for 2 weeks | | MG-63 | Perfusion: ↑ (no significant) ALP activity Perfusion + compression: ↑ ALP activity |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | - | 10 mL/min (peristaltic pump) Constant unidirectional | cyclic compression 0.5 Hz 10% strain | | Human MSC | Perfusion: ↑ proliferation ↑ gene expression of Runx2 ↑ osteocalcin amount Perfusion + compression: ↑ proliferation ↑ gene expression of Runx2 ↑ osteocalcin amount |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | Pore size 500 µm Porosity 75% | No perfusion | 3 Hz + 25 Hz 0.125% strain 15 min on day 5 | | MG-63 | ↑ gene expression of coll1, fibronectin and VEGF-A ↑ non-soluble VEGF protein |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | - | No perfusion | Triangular cyclic compression 5% ± 5% + tension: 5% ± 5% 2 sets/day Set: 1h compression+1h rest+1h tension+2h rest During 4 days | | Human MSC | ↑ matrix deposition |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | Macropore size 384 ± 151 µm (range 150–1000 µm) Strut thickness 67±11 µm | No perfusion | 1 Hz 5% strain 2h/day on day 5, 10 and 15 | | MLO-A5 osteoblastic cells | ↑ calcium content, ↑ collagen content, ↑ cells, ↑ gene expression of coll1, OPN and OCN, ↑ stiffness in compression |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | Porosity 90% ± 2 SD Pore size 250–350 µm | 0.1 mL/min Constant unidirectional (applied 1 week after 2 or 3 weeks in static conditions) | Cyclic compression (applied for 1 week after 2 or 3 weeks in static conditions) ~1.5% strain 1 Hz 1h/day | | Primary rat bone cells | ↑ Number of mineralized nodules ↑ local surface strains in mineralized nodule formation site |
| Hydrogel, L-lactide/dextran with HEMA end groups, seeded with coll1 hydrogel (Ø 8mm, H 4mm) | Macropore size 380 ± 30 µm Porosity 66 ± 1 % Interconnected macropores uniformly distributed | 0.002 mL/min (peristaltic pump) Constant unidirectional | 3 Hz + 25 Hz (15 min at day 5) ~ 0.7% strain | | Primary mouse bone cells | ↑ gene expression of C-fos |

Figures

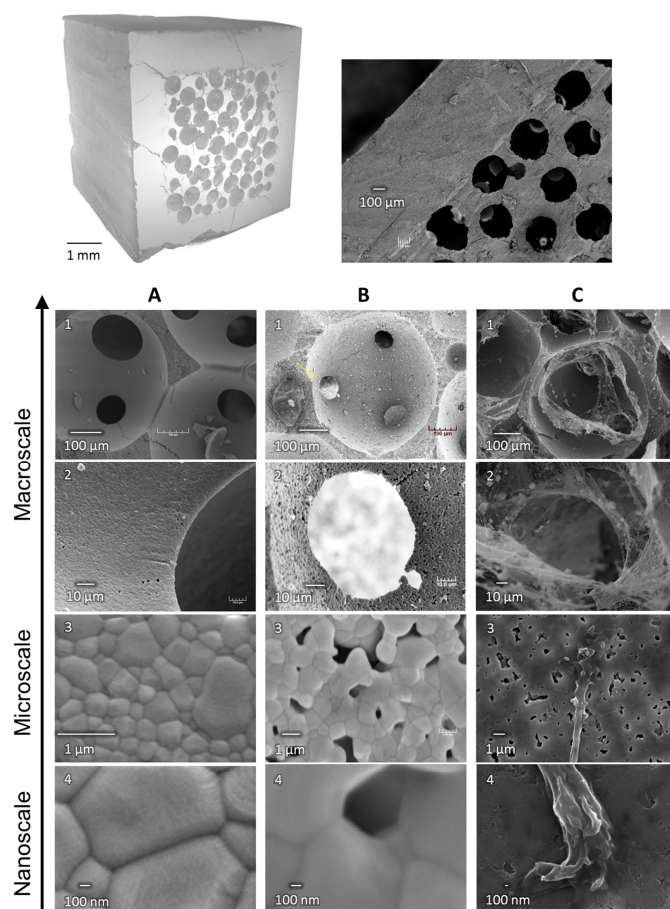
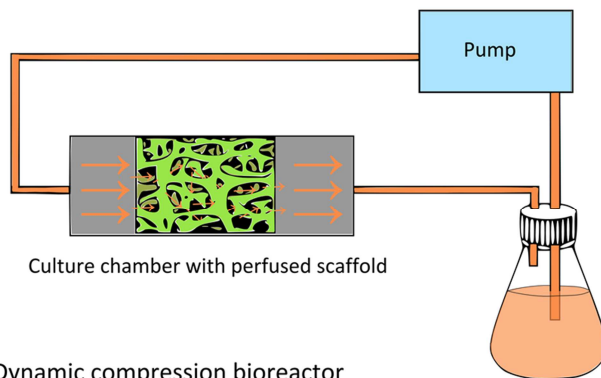


Figure 1. Images from micro-computed tomography (m-CT) and scanning electron microscopy (SEM) of CaP scaffolds manufactured through standard replication of PMMA (Polymethyl Methacrylate) template, (A) without micropore, with micropores (B) before and (C) after 7 days of culture in perfusion bioreactor with mouse calvarial cells (steady flow of 2 $\mu\text{L}/\text{min}$); the yellow arrow on image B1 displays the interconnection pore magnified in image B2.

A Perfusion bioreactor



B Dynamic compression bioreactor

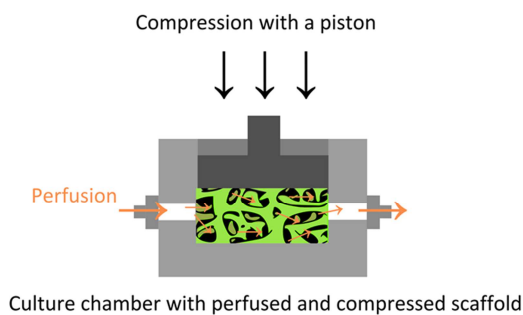


Figure 2. Models of bioreactors. (A) Example of perfusion bioreactor in which the culture medium is forced through the internal interconnected porous network of the seeded scaffold. This can override diffusion limitations and enhance nutrient delivery and waste removal from the cultured cells. The setup includes a medium reservoir, a pump and a tubing system which feeds the culture chamber; (B) Example of perfusion bioreactor allowing the application of controlled dynamic compression to the scaffolds perpendicularly to fluid flow in a dissociated or combined manner.

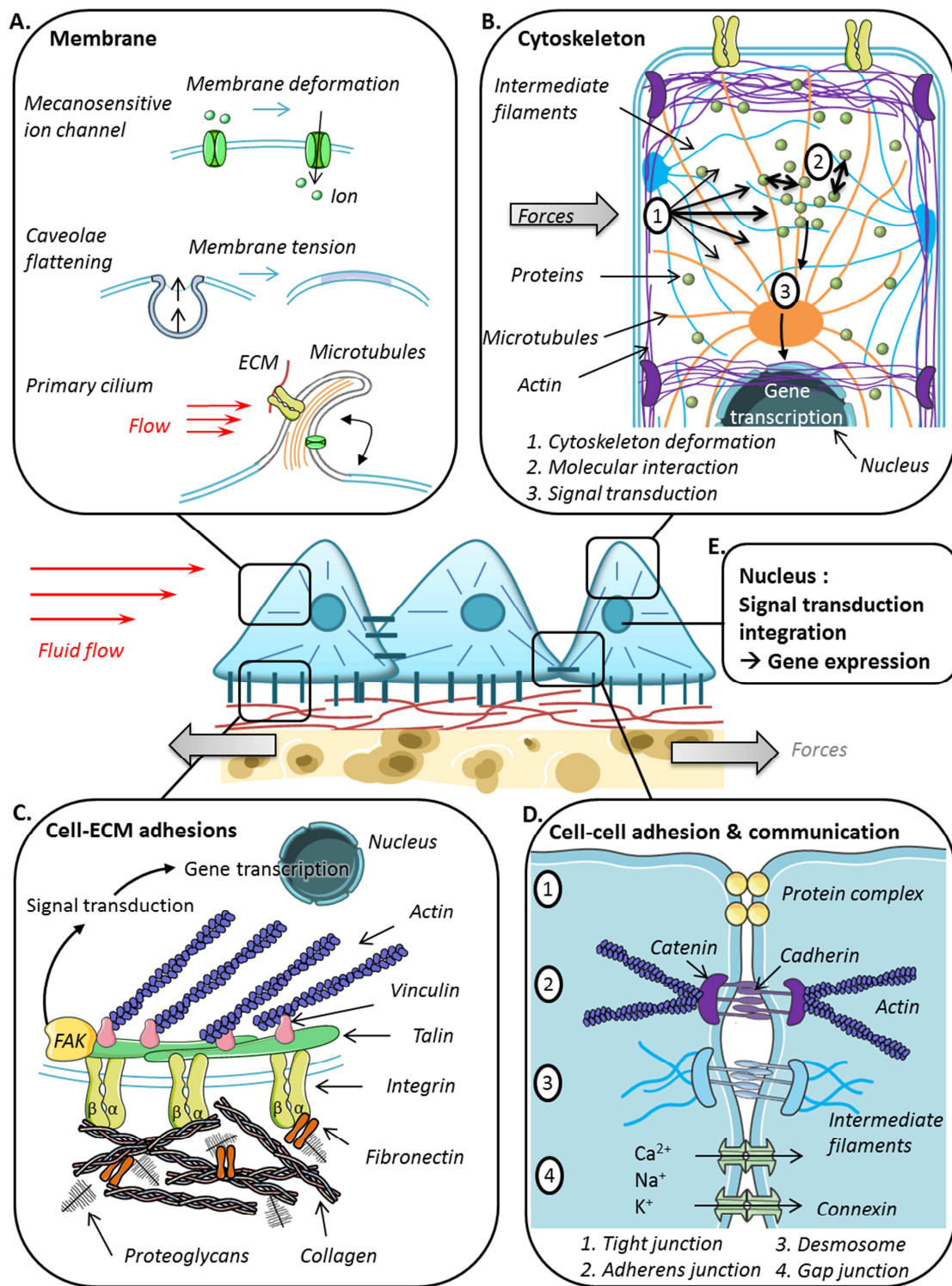


Figure 3. Actors and mechanisms of cellular mechanosensing. Mechanical forces can be sensed and transduced by different means through membrane and/or organelle deformation or cell/ECM interactions.

(A) At the cell membrane level, mechanosensitive ions channels and caveolae (not yet demonstrated in bone cell but in endothelial cells (202)) detect fluid flow or membrane stretching. Ion channels respond by gating, leading to the entry of ions inside cell (203) and caveolae by flattening and activation of specific signaling pathways. Moreover, bone cells possess a single microtubule-based primary cilium which projects from the cell surface and is deflected by fluid flow (206). Its membrane contains receptors and channels which participate in numerous signaling events and play an important role in the mediation of flow-induced calcium deposition by osteoblasts (206).

(B) Mechanical stimulation leads to cell cytoskeletal deformation including actin (stress fiber) reorientation, microtubule polymerization/depolymerisation and intracellular protein displacement (concentration) permitting their interaction and effecting the signal transduction of the original force (207).

(C) As the main cellular mechanical detection mechanism, the transmembrane receptors integrins bind to ECM proteins (e.g. collagen, fibronectin) and activate a mechanosensory protein complex (involving talin, vinculin and focal adhesion kinase (FAK) linked to a kinase cascade system (203).

(D) Cell-cell adhesion, via tight (1) and adherens junctions (2) as well as desmosomes (3), precedes and controls intercellular communication via gap junctions (4), transcellular channels formed by the matching of connexin protein complexes between two neighboring cells. Gap junctions are involved in the propagation of intercellular calcium waves which activate mechanosensitive signal transduction pathways (205).

(E) The multiple transduction signal pathways are integrated by the nucleus and induce changes in gene expression and protein production leading to cell behavior adaptation.

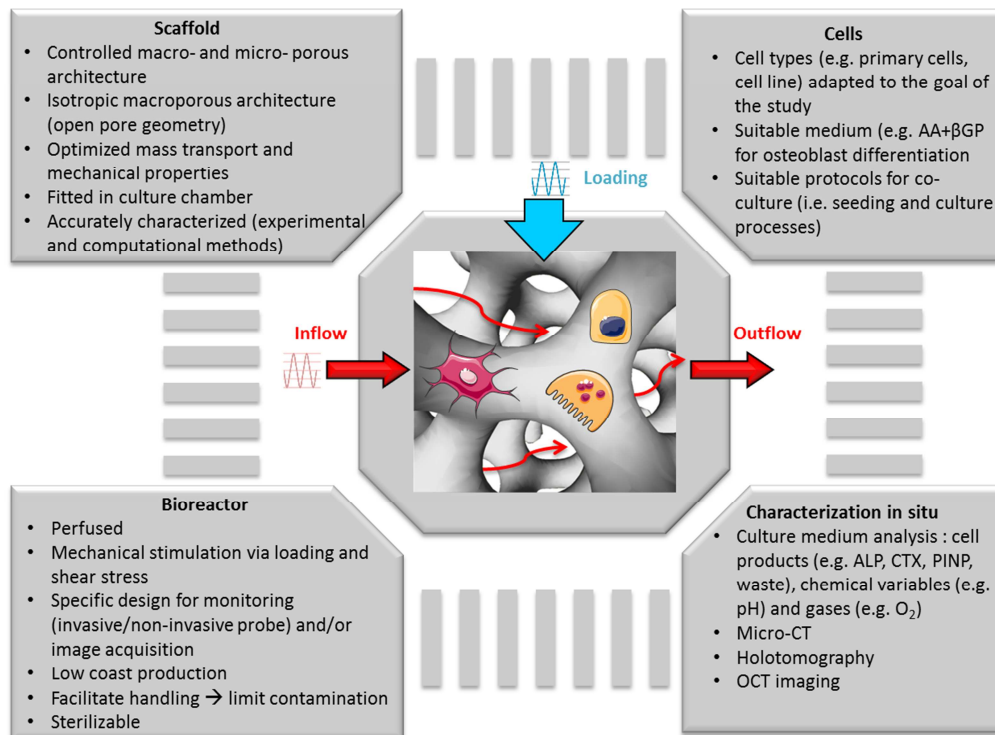


Figure 4. Schematic representation of the key elements of *in vitro* 3D culture model including: cell type choice depending on the goal of the study, main scaffold and bioreactor features needed, as well as characterization tools.

Abbreviations: AA = ascorbic acid, βGP = β-glycerophosphate, ALP = alkaline phosphatase, CTX = carboxy-terminal collagen crosslinks, PINP = amino-terminal propeptide of type I collagen, OCT = optical coherence tomography, micro-CT = micro-computed tomography.